

Development of viral RNA isolation methods from
CymV and TMV-infected orchids and analysis of DNA
isolation protocols for subsequent use in DNA
fingerprinting of orchids.

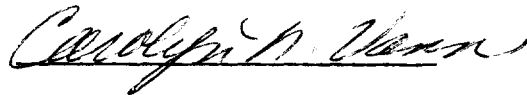
An Honors Thesis (HONRS 499)

by

Jill L. Baker

Thesis Advisor

Dr. Carolyn Vann

A handwritten signature in cursive script, reading "Carolyn D. Vann", written over a horizontal line.

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ABSTRACT

With the presence of the Wheeler Orchid collection at Ball State, we have focused this research on developing methods to isolate nucleic acids from orchids for future studies involving mitigation of virus symptoms and DNA fingerprinting.

In the first step, we isolated RNA from plants infected with the RNA viruses, CyMV and TMV-O. We hoped to distinguish the viral RNA from normal plant RNA. In order to isolate and genetically manipulate it, we would then reintroduce it into the plant to cause mitigation of the viral symptoms. However, there were problems with the isolation of RNA due to the presence of RNases and other degradative enzymes. Although typical procedures were used and precautions were taken to minimize enzymatic activity, most of the RNA isolated was either degraded or degraded within a few weeks of its isolation. Samples degrading more slowly than the others were obtained from using a 4M guanidium thiocyanate procedure. We isolated 100ug of RNA from 6.7 grams of healthy, mature orchid tissue. From mature, virused tissue, 200ug was isolated from 4.5 grams of a plant exhibiting an intermediate form of CyMV and 100ug from 5 grams of a severely virused plant.

With the absence of strict taxonomic guidelines in defining species groups, it is important to develop alternate methods of naming and defining interspecies relationships. In a second group of experiments we attempted to develop a reliable method for DNA isolation yielding high quality DNA from small amounts of plant tissue. We investigated many different procedures utilizing different combinations of cell lysis, CsCl/EtBr density gradients, and phenol/chloroform extractions. Since orchids have numerous phenolic compounds in addition to tough and fibrous tissue and a high concentration of polysaccharides, many procedures failed to deal with all of these factors. The DNA isolated was either degraded or simply could not

be isolated. However, using a 4X CTAB procedure, we obtained between 50 ug and 100 ug per orchid leaf (approximately 4 to 6 grams per leaf). Electrophoresis of these samples showed that the use of early, meristematic tissue resulted in cleaner bands with less degradation. Mature tissue, though, provided as much DNA as early tissue from approximately similar amounts of starting tissues. Digestion of the DNA isolated by restriction endonuclease Eco RI is underway to determine if this relatively simple method will yield sufficient quantities of relatively clean nucleic acids to permit future DNA fingerprinting experiments.

INTRODUCTION

Orchids are endangered plants often put within greenhouses to preserve the variety of species and prevent their extinction. Yet, greenhouse orchids, unlike those in the wild, are vulnerable to certain plant viruses. These viruses, in particular Cymbidium Mosaic (CyMV) and Tobacco Mosaic O-strain (TMV-O), are endemic to greenhouses due in part to crowded conditions often present within greenhouses and the ease with which these viruses are transmitted. Currently, it is estimated that 11% of the orchids within the Ball State Wheeler Collection have either CyMV or TMV-O (Saxon, unpublished). Once a plant has contracted a virus, it will develop necrotic blossom lesions, color breaks, and will eventually die. Thus, it is important to develop a method to mitigate the viral symptoms that are so devastating to the plants' health. Also we would like to disseminate virus free rare species to ensure their survival.

Originally, we were interested in developing a procedure for the isolation of viral RNA from orchids in order to quantify it or isolate it for manipulation. However, more recently we have been interested in utilizing nonsense or interfering RNA for viral symptom mitigation, or in determining the presence or absence of certain satellite RNAs which may affect the severity of viral symptoms.

In addition to our research on orchid viruses, we also are interested in an alternate method of identification of orchids. Traditionally, identification and classification has been through flower morphology which varies with environmental changes. This variability accounts for the discrepancies in the estimated number of total orchid species -- 24 to 60 thousand.

DNA fingerprinting of orchids would provide a more dependable and reliable method of identification and could provide information concerning

evolutionary relationships among various species. This technique is relatively straight forward. Once the DNA is isolated, it is cut with restriction enzymes, electrophoresed, and then analyzed by the Southern method using a labeled probe. After this, a "fingerprint" results which consists of a series of restriction length polymorphisms (RFLPs) that are particular that DNA sample. This method is faster than sequencing the entire genome and makes differences and similarities easy to discern.

Since orchid tissue contains spicules, numerous degradative enzymes and phenolic compounds, we need to find an efficient DNA isolation method. We analyzed different protocols for DNA isolation to find which would provide the highest quality and yield from the smallest amount of starting tissue.

In future fingerprinting experiments, we plan to experiment with a variety of restriction enzymes to determine those which provide the most unique fingerprints. In addition, we will use different probes to see which of those in combination with specific enzymes will produce the most descriptive fingerprint. These methods will be used to establish a database consisting of fingerprints of all of the orchids within the Wheeler Collection. Relationships can be identified between those within the collection and any incoming orchids.

LITERATURE REVIEW

A. The Wheeler Orchid Collection

The Ball State Wheeler Orchid Collection began in 1972 with donations from the Ball family. These orchids were originally collected in the 1940s, 50s, and 60s. In 1981, additional plants, collected from Central and South American jungles were added. This diverse collection is a repository of some of the rarest orchid species in the world. It functions as a species bank by receiving and sending orchid propagules worldwide and as a rescue station for plants seized by the U. S. Customs Service.

With the number of orchids the Wheeler collection receives from Customs, identification of these and those already in the collection can be a problem. The number of orchid species is estimated between 24 to 60 thousand. With the absence of strict taxonomic guidelines in defining the species groups, it is important to be able to categorize and define relationships between and among species. Orchids do not bloom every year and most plants received from Custom's seizures are unidentified and are approximately two years from blooming. It is currently estimated that 20 percent of the plants within the Wheeler Orchid Collection are unidentified.

B. Viruses

With the numerous functions the collection serves, it is important to keep diseased plants to a minimum. The two viruses prevalent among orchids are Cymbidium Mosaic Virus (CyMV) and Tobacco Mosaic Virus - O strain (TMV-O) [1]. These are sap-transmissible RNA viruses which cause necrotic blossom lesions, color break in the bloom, and eventually death of the plant.

These viruses are endemic to greenhouses due to often crowded conditions and the use of contaminated cutting tools on uninfected plants. Once a virus is introduced in a plant, there is no known "cure" for it. In

1989, antibody tests performed on orchids within the Wheeler Orchid Collection showed approximately 11% of the plants within the collection have either CyMV or TMV-O (Saxon, unpublished).

C. Nucleic Acid Isolation

Although there are many published protocols on DNA isolation, we found that many did not account for the numerous phenolic compounds present in orchids. Traditional method included classical CsCl density-gradient centrifugation, gel filtration chromatography, binding to glass powder and precipitation by cetyltrimethylammonium bromide (CTAB), following extraction of DNA by alkaline lysis or boiling [20]. However, traditional procedures often do not account for phenolics or wound-response molecules secreted in response to a cut or break. Orchids also have spicules which will cause the release of phenolics by rupturing cells. We found that a 4X (8%) CTAB solution effectively prevented degradation and provided quality DNA.

Traditional RNA isolation procedures often include lengthy procedures including ultracentrifugation, toxic and/or expensive chemicals like LiCl, guanidinium salts, and cesium salts. There are several rapid isolation procedures involve complex solutions and/or isopycnic centrifugation. For our purposes, guanidium thiocyanate seemed to inhibit the phenolics and RNases present within orchid tissue [2, 11]. This procedure also used a CsCl cushion to further inhibit degradative compounds.

Both of the procedures we used for DNA and RNA isolation needed to be effective on both mature and juvenile tissue either dried or fresh. Due to these constraints, both isolation buffers contained 2-mercaptoethanol. This is an oxidant that inhibits the degradative qualities of phenolics. In addition, both the CTAB (for DNA) and the guanidium thiocyanate (for RNA) helped to inhibit the phenolics. However, we needed to increase the CTAB

concentration to 8% instead of the 2% in the published CTAB procedure which was not strong enough to totally inhibit degradation. (Mark Chase, personal correspondence)

D. RNA Virus symptom mitigation

Much work has been done in developing methods of viral symptom mitigation [1, 4, 9, 10, 16, 17]. This is as important for tomatoes, tobacco and other plants as for orchids. Since dicots such as tobacco are much easier to work with than monocots like orchids, there is more published information on viral symptom mitigation in dicots than in monocots.

The use of nonsense or interfering RNA to mitigate viral symptoms has been very effective [1, 9, 10, 16]. Our initial approach was to use a TI plasmid from Agrobacterium Tumifaciens as a vector to insert recombinant RNA into a viruses plant. This approach is similar to that of Smith et al. 1988 who inserted antisense RNA in place of the viral coat protein sequence in vitro and then re-introduced the RNA into a tomato plant [17]. The antisense RNA was found to reduce expression of the marker polygalactose gene in transgenic tomatoes. However, since monocotyledonous systems are not as easily transformed by TI plasmids, we looked at a variety of approaches in addition to protoplast transformation.

An approach utilized by Carrington et al. 1987, 1989 showed that in addition to the main viral RNA genome, many host cells infected with turnip crinkle virus contain smaller, discreet and sometimes circular RNA strands [1]. One of these types, satellite RNA, has been found responsible for either attenuating or intensifying symptom expression in affected plant hosts. Carrington found an sRNA composed of only the 5' and 3' portions of the RNA genome with the central portion imperfectly presented to exacerbate viral symptoms. This suggested a "blank" genome that contained the origin

of replication and insertion instructions but lacked what information to insert.

Simon, 1988, pinpointed which satellite of turnip crinkle virus (TCV) was responsible for intensifying viral symptoms in turnips [16]. They examined this satellite (sRNA-C) further and found it to be a hybrid molecule. The 3' domain was similar to two 3' domains of the helper virus while the 5' domain of sRNA-C was similar to another sRNA but which was avirulent and had no affect on viral symptoms. From this they hypothesized that the virulency was decided by the 3' domain of sRNA-C. This region was also thought to be important for infectivity and modulating symptom expression. It is not known exactly how the sRNAs interact with the host to create viral symptoms. Work with tobacco mosaic virus has shown a possible involvement of the coat protein in viral symptom production.

Further work by Li, 1989 found defective interfering RNAs (diRNA) associated with TCV which had previously been associated mainly with animal viruses [10]. DiRNA are defective versions of viral RNAs that, unlike sRNAs, have lost essential coding sequences required for independent replication, maturation, or packaging. Whereas sRNAs are infectious of themselves, diRNAs are not and require the presence of a helper virus. One diRNA in particular was found to affect the accumulation of viral RNA and intensified symptoms of its viral helper. Although a diRNA found in tomato bushy stunt virus attenuated viral symptoms.

In Li's work, he used viral isolates not containing the sRNA-C reported previously by Simon that intensified viral symptoms. Also, this diRNA contained the same 3' domain homology to TCV that has been reported for sRNA-C. More recently we have been examining the overexpression of viral coat protein as an antisense RNA encoding the coat protein as a means to

minigate viral symptoms. We became interested in developing a quick and effective procedure to isolate both RNA and DNA from either diseased or healthy plants.

E. DNA Fingerprinting

The key to DNA fingerprinting is its high degree of specificity. With this specificity, it has numerous applications not limited to taxonomic questions. DNA fingerprinting is a definitive test and its power lies in the low probability that different individuals share rare alleles at a number of loci by chance. Once this technique has become more common, data bases for populations can be developed which will insure its statistical accuracy. DNA fingerprinting provides more information than current techniques like HLA and blood group substance analysis which only exclude an individual or group by the presence of differences rather than similarities. The technique of DNA fingerprinting has found to be helpful with all types of cells. DNA fingerprinting has been a deciding factor in many court cases. The first case was in 1985 and involved proving a boy's British citizenship after a visit to his father in Ghana [8]. More recently, the technique has been used to determine paternity cases and criminal cases like rape and murder.

The foundation for DNA fingerprinting was laid by Wyman and White in 1980 with their observation of polymorphic DNA loci characterized by a number of variable length restriction fragments called restriction fragment length polymorphisms (RFLPs) [19]. In 1985, Alex Jeffrey's publication in this area concerned probe sequences that could distinguish these polymorphic DNA loci [6]. His work led the way for many others [3, 6, 13, 18]. He based his probes on random 'mini-satellite' regions in human DNA. These mini-satellite regions are highly repetitive sequences of DNA that tend to

be highly conserved. He used these sequences in comparisons between humans and higher primates and in comparisons of ethnic cultures. He found these profiles to be unique between individuals but shared common loci among cultures or groups.

Later research by Barbara Schall used chloroplast DNA fragments generated by enzyme digestion and common ribosomal DNA as probes for studying relationships among dandelions [7]. She also used human mini-satellite probes generated by Jeffries that showed enough variation among species but were also somatically stable within individual species.

MATERIALS AND METHODS

I. RNA

A. Species

One species that was used was Cattleya Percivaliana v. semi alba 'Jewel'. Orchid tissue samples were described as healthy or virused but we were generally not concerned with species' names.

B. Treatment of Glassware

All glassware, plastic, and solutions needed to be specially treated to remove RNases and to be sterile. In addition, glassware in direct contact with RNA, mainly Corvex tubes and glass homogenizer, needed to be siliconized to prevent the nucleic acids from sticking to the surface.

Siliconizing glassware involved spreading liquid Silicona (Sigma) around the interior of the glassware which was then baked for approximately 36 hours at 170°C.

All other glassware not needing siliconizing was treated overnight with diethylpyrocarbonate (DEPC), an RNase inhibitor. A solution with a 0.1% (1 ul/1ml) concentration of DEPC was allowed to sit overnight inside the glassware and which was then autoclaved on the slow exhaust setting. Eppendorf tubes and pipette tips were treated in a similar manner.

RNases were removed from solutions by addition of 0.1% DEPC. These solutions were allowed to sit overnight and then were autoclaved as long as the chemicals comprising the solution could be autoclaved. This is unstable in the presence of DEPC and thus could not be treated with DEPC [15].

C. Isolation Procedures

Orchid leaf tissue was obtained from mature plants, both healthy and

virused. A mature orchid leaf (approximately 3 to 5 grams) was cut off with a sterile razor blade and weighed. RNA was isolated from fresh tissue or, in some cases, the tissue was immediately put into -70°C to freeze for future use.

In order to isolate RNA, leaf tissue was minced on ice and homogenized at room temperature with 1 ml of Solution D (4M guanidium thiocyanate; 25 mM sodium citrate, pH 7.0; 5% sarcosyl). Solution D was made fresh every month and stored at room temperature. Before use, 0.1M 2-mercaptoethanol was added directly to the solution since it is not as stable as the other chemicals.

After homogenization, the following were added in sequence: 0.1ml 2M NaOAc, (pH 4.0), 1 ml phenol, and 0.2 ml chloroform. The samples were vortexed after each addition and then vortexed a final time for 10s. They were chilled on ice for 15 minutes and centrifuged at $10,000 \times g$ for 20 minutes at 4°C . Following this step, three layers developed: an organic phase consisting of degraded protein, a DNA interface, and an RNA aqueous phase. The RNA was contained within the uppermost phase which was subsequently transferred to an Eppendorf tube.

An additional chloroform extraction was done to remove any lingering phenol traces. To each sample, 400 μl of sterile water, 40 μl 2M NaOAc (pH 4.0), and 400 μl chloroform were added. The samples were precipitated at either -70°C for thirty minutes or overnight at -20°C . DNA was precipitated following centrifugation in a refrigerated mini-centrifuge for five minutes at $10,000 \times g$. RNA was precipitated with isopropanol alcohol at two times the sample volume. The samples were again frozen for either thirty minutes at -70°C or overnight at -20°C and then briefly centrifuged.

The samples were reprecipitated after the supernate was poured off. First the pellet was redissolved in 300 μl of Solution D and one volume of

isopropanol was added. In order to remove further contaminants, samples were again put at either -70°C or -20°C and then briefly centrifuged. The supernate was removed and the pellet washed once or twice in 75% ethanol. The resulting pellet was stored in ethanol at -70°C for extended storage for short term storage at -20°C.

When this procedure yielded degraded RNA, an additional isolation procedure was used. This procedure also used a guanidium thiocyanate isolation buffer (5M guanidium thiocyanate; 50 mM tric-HCL, (pH 7.5), 10 mM EDTA, and 5% 2-mercaptoethanol). This time the solution was not pretreated with diethyl pyrocarbonate due to the reaction between it and the trizma base. Instead it was filtered through a 22 um filter into a previously DEPC-treated and autoclaved sterile solution bottle.

The tissue was harvested in a manner similar to that in the previous isolation procedure. It was minced, on ice, in four volumes of guanidium thiocyanate buffer and homogenized in DEPC-treated glass tissue homogenizer. N-lauryl sarcosine was added to give a final concentration of 4% (w/v) and CsCl to 0.15 g/ml. After dissolving the salt, cellular debris was removed by centrifugation at 15,000 x g, 4°C, for 20 minutes.

The resulting supernate was layered on top of a 1 ml 5.7M CsCl cushion in a Beckman SW 50.1 ultracentrifuge tube. The RNA was pelleted at 100,000 x g for 18 hours at 20°C. The homogenate was carefully removed from the pellet and the pellet and walls of the tube are gently washed a few times with sterile, DEPC water.

The pellet was further dissolved in 10mM tris-HCL, pH 7.5. The solution was made 4% (v/v) with 6M ammonium acetate to precipitate the RNA. Two volumes of EtOH were added. The samples were left either at -20°C overnight or -70°C for thirty minutes [12].

D. Quantification and Electrophoresis

The RNA samples were quantified via a "DNA Dipstick" developed by Invitrogen. The concentrations determined were used to calculate the amount of sample subjected to electrophoresis on either agarose-formaldehyde gels or urea-PAGE gels.

RNA samples were first run on agarose-formaldehyde gels for visualization. The gel consisted of 1.5% agarose (1.05 grams), 60.9 mls of DEPC water, 7 mls 10X MOPS, and 2.1 mls 37% formaldehyde in 70 mls. The agarose and DEPC water were heated to melt the agarose. The solution was allowed to cool to 60°C at which time the 10X MOPS and formaldehyde were added. In some cases, EtBr stock (10mg/ml) was directly added (1 ul/gel) along with the 10X MOPS and formaldehyde. Rosen, however, suggested that the addition of the EtBr directly to the samples immediately prior to loading allowed for better visualization later [14]. Both methods were tried with Rosen's method eliminating additional time spent staining and destaining of the gel prior to visualization.

With the size of gel apparatus used, the lanes could hold up to 35 ug of sample which consisted of the following: 2 uls 10X MOPS, 3.5 uls formaldehyde, 10 uls formamide, 5-10 ugs of RNA (as determined from DNA dipstick), and 5 uls dye marker. These were vortexed and spun briefly to concentrate samples in bottom of eppendorf tube. They were incubated fifteen minutes at 55°C, vortexed, spun briefly, and loaded into the wells. In addition, an RNA ladder and lambda DNA digested with Hind III served as controls on each gel. Although the restricted lambda DNA required no preparation before loading, the RNA ladder was prepared by the same method used for the other RNA samples as denoted above. In all cases, EtBr (1ul/sample) was added to the samples immediately prior to loading on to the

gel. Any excess EtBr that didn't bind to the RNA or DNA maker, migrated off the gel toward the negative pole.

The gels were run at 50 volts in 1 x MOPS buffer for approximately one hour or until the bromophenol blue dye in the marker had migrated approximately 1/2-way down the gel toward the positive pole. (The RNA ran faster than the dye.)

Urea-PAGE gels were used to get better definition of the RNA bands. The samples were run on a polyacrylamide gel consisting of 100 mls of low-salt TBE (57 g acrylamide, 3 g bis-acrylamide, and 480 g urea) brought to total volume of 1 L and stored at room temperature. Immediately prior to pouring the gel, 20 ul of TEMED and 200 ul of ammonium persulfate (1 ug/ml) are added to cause polymerization. To insure full polymerization, the solution was degassed prior to the addition of the ammonium persulfate.

Urea-PAGE gels used a low-salt solution within the gel itself and a high-salt solution as running buffer. The low-salt buffer consisted of 108 g of tris-HCL, 5.5 g borate, and 9.3 g EDTA per litre. The pH was adjusted to 7.6 and was used at the original 10X concentration. However, the high salt solution was also made a 10X concentration but was diluted to 1X prior to use. It consisted of 168 g of tris-HCL, 23.5 g borate (H_3BO_3), and 9.3 g EDTA (pH at 7.6) per litre.

The samples (30 ul total volume) were prepared by adding 1/10 volume of loading buffer (15% Ficoll, 1 mM EDTA, 4% bromophenol blue and xylene cyanol), boiling for approximately 10 minutes, and quickly loading after the addition of 1 ul of 10 mg/ml EtBr. Prior to loading, the lanes are washed out with high salt buffer to prevent the urea from settling. The samples contained 10 to 20 ug of RNA as determined previously. Gels were run at approximately 900 volts for four hours or until the xylene cyanol dye had migrated off the gel.

The samples were run against an RNA ladder which served as a control. It consisted of eight RNA fragments ranging in size from 0.16 to 1.77 Kb in 10 mM HEPES (pH 7.2) and 2 mM EDTA. These sequences were derived from bacteriophage T7 and the rat prolactin gene. Samples run on the gels contained 4 ul of the ladder which was approximately 4 ug of RNA.

II. DNA

A. Orchid Species

Tissues from numerous orchid species were used (Fig. 1). Notation of the particular species used was made for use in subsequent fingerprinting experiments.

B. DNA Isolation

Tissues subjected to DNA isolation were obtained from mature leaf tissue of healthy orchids. A variety of isolation methods were tried. However, the most successful one utilized a 4X CTAB solution as described below.

Fresh leaf tissue, 0.5 to 1.0 g, was broken up in a blender in pre-warmed (60°C) 4X CTAB buffer (8% w/v CTAB; 1.4 M NaCl, 0.2% v/v 2-mercaptoethanol, 20 mM EDTA, 100 mM tris-HCL, pH 8.0). The samples were then incubated at 60°C for thirty minutes. Afterwards, proteins were further denatured with a 24:1 chloroform-isoamyl extraction which was centrifuged (1600 x g) at room temperature to concentrate the phases. The aqueous phase was removed and transferred to a clean centrifuge tube. DNA was precipitated by the addition of 2/3 volume cold isopropanol which was mixed gently to encourage DNA precipitation. At this stage the appearance of the DNA dictated the treatment. It was centrifuged at low speeds if it

appeared flocculent or centrifuged at higher speeds if the DNA was not readily apparent. The recovered DNA was washed in 10 to 20 mls of wash buffer (76% v/v ethanol, 10 mM ammonium acetate) for a minimum of 20 minutes and centrifuged again (1600 x g) for ten minutes. The supernate was poured off and the pellet was allowed to air dry at room temperature.

DNA at this point was usually clean enough to proceed to gel electrophoresis and restriction digests. However, digestion with RNase A in a final sample concentration of 10 ug/ml for thirty minutes at 37°C was often done to insure sample purity. After incubating, the sample was diluted with 2 volumes of TE. Ammonium acetate (pH 7.7) was added to a concentration of 2.5M with 7.5M stock, and 2.5 volumes of cold EtOH was added. DNA was precipitated by gently mixing and centrifuging at 10,000 x g for ten minutes. The pellet was again allowed to air dry and resuspended in TE [5].

Another DNA isolation procedure utilized separate buffers for the tissue homogenization and cell lysis. A third method used only one isolation buffer combining the homogenization buffer and lysis buffer solutions. In both of these methods, the DNA samples were subjected to EtBr/CsCl density centrifugation in gradients with densities adjusted to 1.57g/ml. Centrifugation was performed overnight at 42K.

C. Quantification and Visualization

Some DNA samples were quantified by a "DNA dipstick" from Invitrogen as mentioned for RNA quantification. Others concentrations were estimated by visualization of DNA on a 0.7% agarose baby gel (0.21g agarose, 30 mls 1 x E buffer).

DNA isolated by the previous methods was visualized on a 0.7% agarose gel. The samples were prepared by the addition of marker dye containing

0.25% bromophenol blue and 40% (w/v) sucrose brought to volume in HPLC-grade water.

D. Enzyme Digestion

The DNA was digested with Eco RI. The standard method for digests was 1 Unit of enzyme per 1 ug of DNA per hour. Eco RI had a stock concentration of 10 Units/ul. Thus, a two hour digestion required only 2 uls of enzyme.

To insure that all of the genomic DNA was cut, the digests were incubated for 2 hours at 37°C. The digests were set up as follows: 10 ugs DNA, 2 uls Eco RI, 2.5 uls 10X enzyme buffer (specific to Eco RI), and enough HPLC-grade water to bring the sample to 25 uls.

After two hours, the samples were removed from the 37°C waterbath. Five uls of loading buffer (0.25% bromophenol blue, 40% (w/v) sucrose) were immediately added. The samples were then loaded on a 1.2% agarose gel (1.23 g agarose, 17 mls 1 X E buffer, and 1 ul EtBr). Lambda DNA restricted with Hind III was run as a control. The gel was run at 2 volts for 2 to 3 hours or until the DNA reached within 1 to 2 centimeters of the end of the gel.

RESULTS AND DISCUSSION

I. RNA

A. Isolation of RNA

All of the procedures used included a guanidium thiocyanate isolation buffer. The method by McGookin allowed for the least amount of handling and worked best [12]. His procedure utilized an overnight spin on cesium chloride cushion. The sample was put on this immediately after the initial homogenization. Since RNases are not active in CsCl, they were inactivated at an earlier point in the isolation than other procedures. Even though, the samples did eventually degrade, we were able to obtain RNA from a healthy orchid and two expressing different levels of viral infection, an intermediate and virulent form (Fig. 2). The eventual degradation of the RNA may have resulted from phenolic compounds still present in the samples or from periodic problems we encountered with organic compounds in our water which cause nucleic acid degradation.

In Figure 2, the RNA yields from diseased and non-diseased plants of the same species are shown. In addition, a picture of the electrophoresis is shown. Note that DNA isolated from healthy plants yielded more higher molecular weight RNA. It is not clear whether it was easier to obtain higher molecular weight RNA from these plants or if they have different proportions of various sizes of RNA molecules.

B. Electrophoresis of RNA

Three samples which were the slowest in degrading were subjected to electrophoresis (Fig. 2). Note that some degradation is apparent in the picture in which smears of bands are seen. Prior to optimization of the RNA isolation procedures, we decided to alter our isolation procedure to obtain DNA needed for fingerprinting and genetic manipulation. The RNA obtained in

these procedures was found to be more stable.

II. DNA

A. DNA Isolation

Although a variety of methods were used, the method that seemed to provide the most distinct bands was that described by Doyle and Doyle in 1990 [5]. However, the procedure was modified a bit from the one published. Doyle and Doyle used a 2% CTAB isolation buffer whereas we used an 8% (4X) CTAB isolation buffer (personal communication, Mark Chase). Due to the presence of phenolic compounds and DNases, we believed that the 2% solution was not concentrated enough to effectively neutralize all of the degradation-causing compounds within the orchid tissue. (Doyle and Doyle mention in the concluding paragraph of this paper that some plants work better when a higher percentage of CTAB is used in the isolation buffer.)

B. Electrophoresis

Only DNA isolated from Laelia anceps Hautesca was run on a gel mainly to check the quality and concentration of the sample. (Fig. 3) Concentrations on the gel in Fig. 3 range from 50 ug to 100 ug per sample. Although, the band in lanes 2 and 3 in figure 3 are running slightly faster than the last band (23kb) of the lambda marker, we believe it is chromosomal DNA. There is some polysaccharide contamination still present in the wells. RNA is degraded and running faster than the lambda marker. The smears between the chromosomal DNA and RNA could be degraded DNA and/or satellites or plasmid DNA. Digestions are underway to confirm that this method of isolation will be useful for obtaining DNA for fingerprinting.

CONCLUSION

A quick and efficient method for nucleic acid isolation from orchids was determined. This method was able to provide high quality DNA or RNA from a small amount of starting tissue either dry or fresh, mature or juvenile. The 4X CTAB procedure gave a good yield of undegraded DNA that was able to be digested for use in future experiments concerned with genetic engineering or DNA fingerprinting.

Figure 1

These are the orchid species that were used for the DNA isolations. Those species from which DNA was successfully isolated are in Figure 3.

Figure 1

Cattleya Skinneri "Black Devil"	Laelia Purpurata v. Venosa
Cattleya Labiata v. Semialba "Preyon"	Laelia C Elegans v. "Ecuilad"
Cattleya Deckeri	Laelia Purpurata v. Soerulea
Cattleya Guatamaliensis "La Libertad"	D O Crente
Cattleya Aurientaca "Red Stripe"	
Cattleya Skinneri	
Cattleya Chocolate Drop x Ctna Keith Roth	
Cattleya Aurantiaca Thurniers	
Cattleya x Guatamaliensis Purple	
Cattleya x Guatamaliensis Orange	
Epidendrum Anceps	
Rhyncholaelia Digbiana 'Mrs. Chase'	
Broughtonia Sanguinea	

Figure 2

Total RNA Yield and Electrophoresis

The table shows the starting tissue compared to the amount of RNA obtained. The last row shows the amount of RNA that was run on the gel pictured in the bottom of the page.

The gel is as follows: Lane 1, RNA ladder; 2, healthy; 3, intermediate; 4, virulent. This is a 1.2% agarose-formaldehyde gel. The RNA samples were isolated from a 4M guanidium thiocyanate procedure utilizing a CsCl cushion and 19 hour centrifugation.

Figure 2

	Starting Tissue	Total RNA yield	Amount on gel
Healthy	6.7 grams	100 ug = .5 ug/ul	10 ug
Intermediate	4.5 grams	200 ug = 1.0 ug/ul	20 ug
Virulent	5 grams	100 ug = .5 ug/ul	10 ug

1.77 kb
 1.52 kb
 1.28 kb
 0.78 kb
 0.53 kb
 0.40 kb

 0.28 kb
 0.16 kb



Figure 3

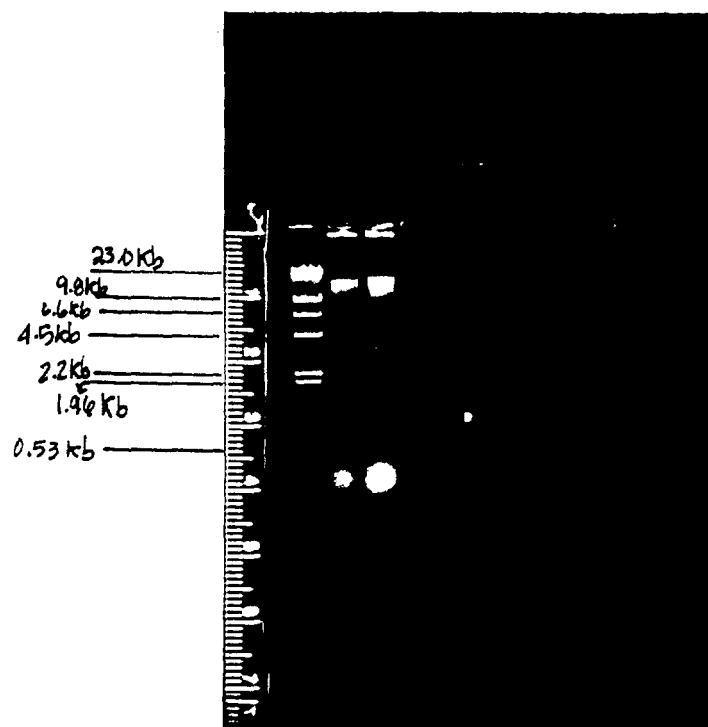
Total DNA Yield and Electrophoresis

The table highlights the species that were successfully isolated using Doyle & Doyle's procedure.

The gel is as follows: Lane 1, lambda/Hind III; 2, 10 ul; 3, 20 ul. This is a 0.7% agarose gel. It was run at 50 volts for 1 1/2 hours. The chromosomal DNA is running slightly faster than the 23 kb band of the Lambda marker.

Figure 3

Species Name	Starting amount	Total DNA yield	Amount on gel
Laelia Anceps Hautesca	1 leaf	50 ug = .05 ug/ul	10 ul / 20 ul
Laelia Anceps 'Jalapa	1 leaf and "bud"	100 ug = .1 ug/ul	-----
Laelia Anceps 'Jalapa	1 leaf	100 ug = .1 ug/ul	-----



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